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(54) Title: METHODS AND COMPOSITIONS FOR BINDING ENDOTOXINS

(57) Abstract

Disclosed are methods and compositions for binding endotoxins useful for the removal or detection of endotoxins, involving amidine moieties. The amidine moieties are preferably provided by True Blue or 4,6-Diamidino-2-phenylindole (DAPI). A preferred support is POROS^{R}. The compositions may be used in treatment of conditions such as septic shock.

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METHODS AND COMPOSTIONS FOR BINDING ENDOTOXINS

BACKGROUND OF THE INVENTION

Lipopolysaccharide (LPS), also known as endotoxin, (the terms are used interchangeably herein) is expressed in the outer membrane of Gram negative bacteria. The molecule consists of a hydrophobic Lipid A region linked to a core oligosaccharide and an outer polysaccharide chain. Production and release of lipopolysaccharide occurs in patients infected with gram negative bacteria; this results in the stimulation of an excessive immunogenic reaction and may lead to septic shock. Septic shock leads to a cascade of host responses to lipopolysaccharide, importantly, the elaboration of proinflammatory cytokines including tumor necrosis factor and various interleukins by monocytes, ultimately manifesting in the shock syndrome. In the U.S. this type of infection may be responsible for approximately 100,000 deaths annually.

Endotoxins are especially problematic in the pharmaceutical industry where federal regulatory agencies require that the production and purification of biologically active proteins be monitored to confirm that processes are functioning within established boundaries. Endotoxins often are introduced into a process from contaminated raw materials or from source material. For example, endotoxins may be introduced from buffers, salts, chromatographic media, cell culture, fermentation additives, and/or water. Contamination by endotoxins is particularly common when expressing recombinant proteins in bacteria. Endotoxins vary in size depending on whether divalent ions and surface active agents are present. The location of a product in the hosts and the mechanism of cell disruption can have a significant impact on the amount of endotoxin released with a product. If cell disruption methods change, as they often do during scale-up fermentation, then the ability of the purification process to remove and detect endotoxins must have sufficient capacity to accommodate variability. Because of the wide size distribution of endotoxins,

methods that rely on size will be unreliable unless all of the factors that influence size distribution are well controlled.

The amphiphilic lipid portion of lipopolysaccharide, termed Lipid A, elicits most of the toxic effects of lipopolysaccharide, and therefore represents the toxic center of the endotoxin. Thus, one therapeutic approach aimed at the abrogation of endotoxicity has been directed towards the neutralization of lipid A activity. The antibiotic polymyxin B (PmxB) binds with relatively high affinity to the Lipid A portion of lipopolysaccharide and results in the inability of lipopolysaccharide to invoke the inflammatory response in macrophages and monocytes, its primary site of action. Since Lipid A partial structures can act in a similar fashion to PmxB and inhibit the response to LPS in monocytes and macrophages, it has been postulated that the Lipid A region would be an appropriate therapeutic target for drug development. A recent study by Rustici et al. examined the ability of synthetic peptides to mimic the action of PmxB in binding to Lipid A, thus acting as inhibitors of the inflammatory response; they found that a contribution from both the hydrophobic region of synthetic peptides together with some cationic character were required for optimal activity in the binding of these peptides to Lipid A. The use of PmxB is limited, however, because it is too toxic to use clinically.

Affinity supports using PmxB coupled to agarose or polystyrene beads have been used for the removal of endotoxins from solution. However, these supports have several disadvantages. First, these supports may leach the PmxB (which is toxic); second, the peptide has a limited lifespan so activity is lost with time; and, finally, PmxB is only able to recognize certain serotypes of endotoxins with significant affinity.

Similarly, investigators have studied the effect of pentamidines on endotoxins. They suggest that, in the case of pentamidine, the affinity for lipid A was based on the presence of two strongly basic amidines which are protonated at physiological pH, separated by a distance that is

commensurate with the inter-phosphate distance of lipid A. The affinity of pentamidine for lipid A was found to be even greater than that of PmxB, and was examined at therapeutic concentrations on the induction of several cytokines by whole lipopolysaccharide in cultured human peripheral blood mononuclear cells. Although modifications to the structure of pentamidine were suggested to investigate desirable structural properties, such modifications would be based upon the assumption that increasing the length of the internitrogen bonds on the amidines would cause increased affinity of the modified structures. See David, S.A. et al., Biochim. Biophys. Acta. 1212; 167-175 (1994). To date, applicants are unaware of any further information on modified pentamidines and/or their binding to LPS.

Thus, in view of the problems associated with the presence of endotoxins in pharmaceutical preparations, there is a need for rapid and accurate process monitoring in all stages of pharmaceutical processing. Because cell growth and biosynthetic activity vary from batch to batch, it is necessary to monitor the production of contaminants frequently. There is also a need for useful therapeutic preparations for the treatment of hosts with high levels of endotoxins, as well as for diagnostic kits and preparations for detecting the absence, presence, or concentration of endotoxins.

SUMMARY OF THE INVENTION

Accordingly, the present invention is directed to affinity supports and methods for the detection or removal of endotoxins which substantially obviate one or more of the problems due to the limitations and disadvantages of the related art.

Additional features and advantages of the invention will be set forth in the description which follows, and in part will be apparent from the description or may be learned by practice of the invention. The objectives and other advantages of the invention will be realized and attained

by the process and compounds particularly pointed out in the written description and claims hereof.

To achieve these and other advantages, and in accordance with the purpose of the invention, as embodied and broadly described, the invention relates to molecules capable of binding to endotoxins of various serotypes and methods for detecting and/or removing endotoxins from a sample.

The invention relates to a method for removing lipopolysaccharides from a sample by immobilizing an amidine containing moiety to a solid support such that it retains its ability to bind to lipopolysaccharides, and introducing the sample to the support under conditions sufficient for the lipopolysaccharides, if present, to form a complex with the amidine containing moiety.

In other embodiments, the invention relates to detecting the absence, presence or concentration of a lipopolysaccharide in a sample by contacting the sample with a detectable modified amidine containing moiety. If lipopolysaccharide is present, it will form a complex with the detectable moiety. Thus, the methods are both quantitative and qualitative, and may, in various embodiments, be incorporated into diagnostic or therapeutic applications, such as to remove or detect lipopolysaccharide in a sample.

The invention further relates to an apparatus and a kit including an amidine containing moiety which has the ability to bind to lipopolysaccharide immobilized on a solid support for the removal from, or detection of, lipopolysaccharide in a sample. Additionally, the modified amidine moieties may be used in solution, provided that the complex formed has at least one physical property by which said complex can be separated or identified.

The amidine containing moieties which bind to endotoxins may also have a bacteriocidal effect.

The invention encompasses pharmaceutical compositions containing an amidine containing moiety which binds to endotoxins with a high affinity, as well as any desired fillers, adjuvants or stabilizers.

The invention further contemplates methods for the treatment or diagnosis of endotoxin contamination using amidine containing moieties which bind to endotoxins with a high affinity. In these embodiments, the compositions containing modified amidine containing moieties may be introduced to a host in a therapeutically effective dose, or used to detect or remove endotoxins from fluids removed from the animal.

In yet other embodiments, the claimed invention relates to methods for purifying a sample preparation, for example, recombinantly produced proteins.

The claimed invention may be used in methods, apparatus, kits and compositions for the detection or removal of any phosphate-containing molecule, as well methods, compositions and apparatus for diagnostic or therapeutic applications in specimens having phosphate containing molecules. However, for clarity, the invention will be discussed herein as it relates to lipopolysaccharides.

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory and are intended to provide further explanation of the invention as claimed. The accompanying drawings are included to provide a further understanding of the invention and are incorporated in and constitute a part of this specification, illustrate several embodiments of the invention and together with the description serve to explain the principles of the invention.

DESCRIPTION OF THE DRAWINGS

Figure 1A depicts the removal of endotoxins on various columns having True Blue immobilized on a POROS™ support.

Figure 1B depicts an SDS-PAGE analysis of unbound globulins to resin.

Figure 2 depicts the recovery of LPS (labelled with FITC) on a POROS™-True Blue support

Figure 3 depicts the removal of LPS in the presence of various proteins on POROS™-True Blue columns.

Figure 4 depicts the Frontal analysis of POROS™-True Blue

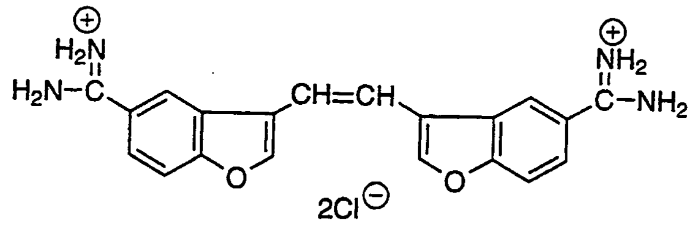
DETAILED DESCRIPTION

Reference will now be made in detail to the present preferred embodiments of the invention.

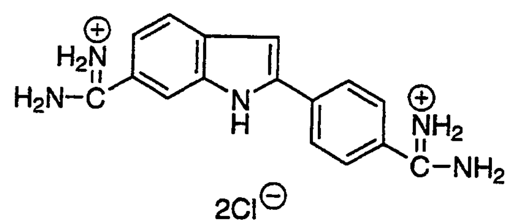
As embodied herein, the method of removing lipopolysaccharides from a sample solution initially involves immobilizing an amidine containing moiety to a solid support such that the moiety retains its ability to bind to lipopolysaccharides. The sample solution is then introduced to the support, and, any lipopolysaccharides present will bind to the amidine containing moiety, thus forming a complex immobilized on the support.

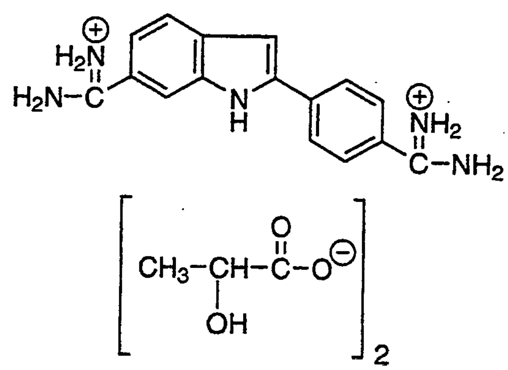
The modified amidine containing moieties of the invention are modified for attachment to a support while maintaining their ability to bind lipopolysaccharide or phosphate containing compounds such as DNA or RNA. Prefererred modified amidine containing moieties are modified pentamidines such as True Blue and DAPI. True Blue and DAPI are commercially available through Molecular Probes. (True Blue and DAPI); SIGMA CHEMICAL CO. (TB and DAPI).

True Blue (TB) has the formula:



DAPI is a compound having one of the following the chemical formulae:





The applicants have determined that True Blue is particularly useful for practice of this invention because it has a higher affinity than pentamidine for binding endotoxins and has a broader specificity than PmxB for the different serotypes of LPS. Additionally, True Blue is flourescent, thereby facilitating detection and quantitation in a suitable assay. Finally, True Blue contains a double bond which can be utilized for immobilization to a solid support in a chemically stable manner. This is particularly advantageous because detoxification requires conditions which would cause leaching of PmxB and other compounds immobilized by amidine linkages.

Similarly the applicants have determined that diamidino-2-phenylindole (DAPI) is a preferred compound for use in this invention because it is fluorescent and has a higher affinity for LPS than does PmxB. The amidine moieties preferably bind to the phosphate structure in the lipopolysaccharide.

Supports to which the modified amidine containing moieties may be immobilized include any solid support which does not interfere to a significant extent with the activity of the modified amidine containing moieties. Commonly used supports include, but are not limited to, controlled pore glass, silica, silica gel, chips, membranes, polystyrene based supports, glass fibres, frits, paper filters, magnetic beads and magnetic particles. The support may be porous or non-porous depending upon the desired application of the invention. Preferably the support is suitable for perfusion chromatography, such as POROS™, available from PerSeptive Biosystems, Inc. Preferably, the amidine containing moieties are immobilized to the support by double bond linkages, rather than amidine linkages.

Samples which may be utilized in the claimed invention include water, pharmaceutical preparations, body fluids, including, but not limited to, blood, serum, plasma, urine cerebrospinal fluid, saliva, sweat, semen, vaginal fluids, amniotic fluid and ascites fluid. Additionally, the claimed invention may be used in the pharmaceutical industry for e.g. removing lipopolysaccharide from water or other fluids used in pharmaceutical processing. The invention is particularly useful for removing endotoxins from preparations derived from recombinant proteins.

The claimed invention is suitable for removing endotoxins from solutions. For example, on a small scale, a sample solution may simply be exposed to the modified pentamidine moieties of the invention immobilized on a solid support thereby removing endotoxins as the solution passes by the support. One may select the parameters of the systems, e.g. length of support, desired degree of purity, depending on the characteristics of the solution to be purified. As the

solution passes the support, the endotoxins, if present, will bind to the amidine containing moiety and be retained on the support.

In other embodiments, it is not necessary that the amidine containing moiety be immobilized on a support. For example, one may merely introduce the moiety into the solution to be analyzed, and remove any complex formed by the binding of the modified amidine containing moiety to the endotoxin by any means known in the art, e.g. filtration, chromatography, sedimentation etc. It is preferable to use amidine moieties having a high affinity for lipopolysaccharide. The methods may also be useful to inactivate lipopolysaccharides.

Additionally, such amidine containing moieties may have bacteriocidal properties and may be used as such, or as an antibiotic.

It is preferable to immobilize the modified amidine containing moiety on a support suitable for perfusion chromatography, such as POROS™ available through PerSeptive Biosystems, Inc. The invention may be useful as a means to remove endotoxin from solutions. Supports according to the invention may be used in any format, for example, a chromatography column, discs, chips (i.e. for surface plasma resonance detection).

Such affinity supports may have application as a potential therapeutic in cases of reperfusion ischemia injury. Patients expressing symptoms of endotoxic shock (septicemia) may be gavaged with the support, which would then remove or inactivate the lipopolysaccharide in the gut, which is the major site of absorption This may reduce the level of lipopolysaccharide load in the host system prior to reperfusion and hence provide increased chances for survival.

In other embodiments, the methods of the invention are to be used to detect the absence, presence or concentration of lipopolysaccharides in a sample. In these embodiments, the sample is contacted with a detectable, amidine containing moiety, such that, if lipopolysaccharides are present, they will form a complex with the amidine containing moiety, thereby indicating the

absence, presence or concentration of the lipopolysaccharide. A detectable moiety, as used herein, is any moiety capable of detection, suitable for use in the claimed invention, including, but not limited to: enzymes, fluorophores, chromophores, radioisotopes, electrochemical moieties and chemoluminescent moieties. Such detectable moieties can be readily conjugated to the amidine containing moieties using any technique know in the art, however, should not interfere with the ability of the modified pentamidine to bind to the lipopolysaccharides. The invention contemplates that the detectable moiety may be intrinsic to the moiety.

The claimed invention may be embodied in an individual apparatus or kit which can be easily used in a wide range of diagnostic tests. The kit may optionally be disposable. Alternatively, the invention contemplates an apparatus or kit useful to remove lipopolysaccharides from a solution. Thus, in one embodiment the invention can detect the presence of endotoxins in solution, and can be used as a component of a quality assurance system for any solution. For example, one may wish to periodically sample production batches to test for impurities such as endotoxins. Additionally, the invention may be incorporated into the upstream processing not only to test for the absence, presence or concentration of endotoxins, but also to actually remove contaminants at any stage of the processing.

It is additionally contemplated that the invention encompass pharmaceutical or diagnostic preparations. For example, a pharmaceutical preparation comprising an amidine containing moiety capable of binding to endotoxins, may be introduced into a host organism. The amidine moieties may then bind to the endotoxins thereby rendering them non-toxic. When immobilized to a support, the amidine containing moieties may be used as part of a dialysis type apparatus to remove fluids from a host organism, purify them on the support, and reintroducing the purified fluid into the organism.

Diagnostic apparatus and kits are also encompassed within the scope of the claimed invention. Thus, in some embodiments, one may immobilize the amidine moieties on a support, such as a biosensor, thereby allowing the capture of lipopolysaccharides. Such devices may be used to either remove lipopolysaccharides from a sample, or to detect or quantitate lipopolysaccharide levels in solution, e.g. in serum from patients suspected of having sepsis, in pharmaceutical analyses for validation purposes, or for detection of pyrogenic quantities of LPS in preparations for administration to humans or other animals. It is also contemplated that the support be used in the form of a cartridge or chip. Additionally, one may, for example, by loading a portion of a hypodermic needle with detectable modified pentamidine moieties, quickly and easily detect the presence of endotoxins. Similarly, depending on the detectable moiety, one may perform qualitative or quantitative analyses rapidly.

Preferred embodiments of kits for detecting the presence, absence or concentration of lipopolysaccharides may be configured to detect clinically relevant lipopolysaccharides' in biological samples.

EXAMPLES

1. <u>The specificity of the ligand true blue , immobilized on a column for the affinity selection of endotoxins</u>.

Batch mode measurements of selectivity by test and control resins.

2 tests and 1 control resin were made.

Resin [1]=45mg TB/100mgs POROS

[2]=1mg TB/100mgs POROS

[3]=POROS treated as for [1] and [2] in the absence of TB

After washing 30µl of a dilute slurry of these resins extensively in lipopolysaccharide-free water, specific binding of lipopolysaccharide was assessed by incubation of these resins with know

quantities of lipopolysaccharide. Unbound lipopolysaccharide was measured using the Limulus Amoebocyte Lysate (LAL) Assay.

As can be seen (Fig. 1a) the greatest reduction in lipopolysaccharide was observed in the presence of resin [1]. Resin [2] exhibited little or no removal of endotoxin when compared to resin [3] or control [C, lipopolysaccharide incubated in the absence of resin]. At the IC_{50} for endotoxin measurement, resin [1] appeared to reduce the level of lipopolysaccharide by 20-fold relative to the controls.

In order to assess the selectivity of the resin with respect to a commercially important protein preparation, 30µl (~600µgs POROS) of each of the resins were incubated under identical conditions to those above with 30µgs of a crude Gamma globulin preparation (Sigma Chemical Co.). After incubation (90min/RT) the resins were removed by centrifugation and the free protein determined by SDS-PAGE of a small aliquot of the supernatant (5µgs protein per track). The protein bands were detected by coomassie staining (Fig. 1b). Track 1=molecular weight standards.

Track 2=Globulins incubated - Resin

Track 3=Globulins + Resin [1]

Track 4=Globulins + Resin [2]

Track 5=Globulins + Resin [3]

No significant binding of Gamma Globulins was observed by any of the resins under the conditions used.

Binding of lipopolysaccharide by resins in a column (2.1 x 30mm) format.

The ability of each of the resins to bind FITC-lipopolysaccharide was determined under a number of different conditions. Direct comparison was made using 50mM tris, pH 7.0, monitoring elution of bound material by fluorescence detection. As can be seen (Fig. 2) maximal

binding of the labeled material was observed with resin [1], levels of binding by resin [2] were slightly reduced while binding to control resin was less than 30% that of [1] under the same conditions.

Recoveries of proteins with respect to FITC-lipopolysaccharide by Resin [1] in a column format.

FITC-lipopolysaccharide was coincubated with the proteins indicated in Fig. 3. Material was injected and run in 50mM Tris, pH 7.0. Material bound to the column was eluted using 1N NaOH. The large fluorescence peak observed in the void apparently represents uncoupled FITC since LAL assay of this material revealed little endotoxin activity (data not shown). The chromatograms show that the column has highly selectivity for lipopolysaccharide in the presence of BSA (C), Myoglobin (D) and Gamma Globulins (E). In the presence of lysozyme, lipopolysaccharide recovery with respect to control (no protein) was similar. However, significant binding of lysozyme to the column was observed. While BSA, Myoglobin and Gamma Globulin binding to the column was not significant there was a slight reduction in the efficacy of the column at removing lipopolysaccharide from these mixtures (presumably due to binding of lipopolysaccharide to these proteins). Optimization of pH and salt conditions may reduce the levels of unwanted non-specific binding. In this regard we have observed a slight increase in the capacity of the column for lipopolysaccharide if the buffer was changed to 20mM phosphate, pH 7.0 (data not shown).

Resin [1] removes> 95% lipopolysaccharide from solution

A 2.1mm x 100 mm column was packed with [1]. lipopolysaccharide activity in an aliquot of FITC-lipopolysaccharide was measured prior to injection over the column and in the void (prior to NaOH elution of bound material).

2000EU/ml were run over the column.

75EU/ml were present in the void volume.

This represents 96% removal of lipopolysaccharide under these conditions.

Frontal analysis of the Column

Repetitive injections of lipopolysaccharide (055:B5 serotype; 100µl/injection of 0.2mg/ml lipopolysaccharide containing FITC-lipopolysaccharide as a marker) were made and fractions of the void volume collected. The frontal is shown (Fig. 4). Calculation of the saturation of the column by consecutive injection suggests that the capacity of the column (2.1 x 100mm) is greater than 400µgs lipopolysaccharide. Subsequent elution of the column resulted in a large fluorescent peak corresponding to the bound material.

The affinity column tested appears to exhibit selectively for endotoxin even when running at fairly high flow rates. The capacity of the column is not quite as high as the "theoretical" value (5mg/ml) for the Affi-prep polymyxin B support sold by BioRad. However, the column does have the advantage that the True Blue should not leach off with use, the columns can be run at higher pressures and flow rates and can be sanitized by treatment with 1N NaOH without significant loss of activity.

2. <u>The use of amidine containing moieties to bind a variety of different serotypes of lipopolysaccharide (endotoxin).</u>

In free solution these moieties were shown to exhibit higher affinity for lipopolysaccharide than polymyxin B. Immobilization of these ligands to a solid support would enable the affinity purification (or removal) of endotoxin from solution. This was demonstrated using a member of this class of compounds with the structure shown below (Fig. 1A). Other candidates with demonstrated abilities to inhibit lipopolysaccharide in the LAL assay are also shown (Fig. 1B and C). The ligand (1A) was immobilized onto POROS. POROS treated identically, except in the absence of the ligand was also synthesized as a control. Figure 2 shows the effect of preincubation of the resins with a known quantity of endotoxin on recovery of endotoxin assayed

using the LAL assay. lipopolysaccharide incubated in the absence of resin was included as a control. Support in the absence of the ligand exhibited only marginal removal of lipopolysaccharide when compared to a control sample which was incubated in the absence of resin (Fig. 2). However, ligand 1A coupled to POROS resulted in a significant shift in the dilution curve for lipopolysaccharide, suggesting removal of endotoxin from solution.

Studies in our group have shown that the amidine groups are critical for optimal interaction between these molecules and lipopolysaccharide since none of the amine-containing compounds or amidines tested were capable of inhibiting the lipopolysaccharide-mediated color change in the LAL assay. Since compound 1B has been shown to bind tightly to DNA through interaction between the amidines and the phosphate groups of the ligand it would seem that the interaction with lipopolysaccharide would be via the phosphate groups present on the Lipid A region of the latter. Indeed competition assays examining the ability of these ligands to compete for binding to lipopolysaccharide, have indicated that compound 1C and polymyxin B bind to the same site. Compound 1C exhibits higher affinity in the assay than polymyxin B. Based on additional data from the LAL assays, it would appear that the rank order of potency for inhibition of the effects of lipopolysaccharide for these compounds is; 1A>1B>1C. Compound 1B has demonstrated activity against the following serotypes of endotoxin in the LAL assay; 055:B5, 0113:H10, and Salmonella abortus equii.

3. <u>A tandem column set up consisting of an anion exchange plumbed above a reversed-phase column was employed to assess the interaction of lipopolysaccharide with PmxB.</u> PmxB eluted with a retention time of 5.7 min (~ 30% acetonitrile concentration) when injected directly onto a reversed-phase column (Fig. 1b, trace A). When the same concentration of PmxB was passed over the anion-exchange column, washed with 10 CV of the equilibration buffer and then eluted onto the reversed phase column, the PmxB peak was not seen (Fig. 1b, trace B) indicating that

PmxB does not bind to the anion exchange column. However, co-incubation of lipopolysaccharide with PmxB resulted in the recovery of peak from the reversed-phase column corresponding to the retention time of PmxB (Fig. 1, Trace D). PmxB was retained on the anion exchange column only in the presence of lipopolysaccharide, suggesting that lipopolysaccharide was still able to bind to the ion exchanger in the presence of this ligand.

The effect of varying the wash volumes passed over the lipopolysaccharide-PmxB complex captured on the anion exchange column prior to elution of PmxB onto the R2 column was investigated. Quantitation of the amount of PmxB eluted off the reversed-phase column (Fig. 2a) demonstrated a relationship between column 1 wash volumes and the subsequent recovery of PmxB (Fig. 2b). PmxB recovery was reduced with increasing wash volume such that a 50% reduction in initial peak height was obtained at 42 CV washes over column 1. This reduction in peak height was not a result of desorption of lipopolysaccharide from the ion exchanger since recovery of FITC-lipopolysaccharide under similar wash conditions showed no significant reduction between 10 and 80 Cvs (data not shown). Since the PmxB was incubated in the presence of lipopolysaccharide to equilibrium, loss of ligand must be a factor of the dissociation rate from its target which would be dependent on time, dilution and association rate constants. The time and dilution factor should be constant under similar conditions; the loss of ligand bound to its target under these conditions should thus be directly related to its dissociation rate (k_{-1}).

To further test whether this model could be used to obtain dissociation rates, we investigated the binding of several known ligands for lipopolysaccharide. A decapeptide (IKTKKFLKKT), shown to bind lipopolysaccharide with lower affinity than PmxB was eluted with a 50% reduction in binding to lipopolysaccharide occurring at ~ 10 CV (Fig. 2b). The corresponding 9-mer (lacking amino terminal isoleucine) resulted in a 50% reduction of peak height for this ligand at approximately 15 Cvs, indicative of a slightly higher relative affinity for

lipopolysaccharide. Pentamidine, reported to bind to lipopolysaccharide more strongly that PmxB, exhibited 50% washout at 46 CV (Fig. 2b). The rank order of the dissociation rates of these ligands from lipopolysaccharide, therefore, was determined to be:

Pentamidine<PmxB<9-mer<10-mer

Empirically, the relative off reaction constants obtained by the tandem column method based upon recovery of ligands from the lipopolysaccharide-ligand complex after varying the wash time correlates well with the rank order of the published values for their affinities for this target.

In addition to demonstrating the binding of ligands of lipopolysaccharide, the site of binding on lipopolysaccharide could be deduced from competition assays. It had previously been shown that PmxB, the 10-mer and pentamidine are capable of binding at this site, a competition assay between PmxB and the 9-mer was carried out. lipopolysaccharide was incubated in the presence of a fixed concentration of the 9-mer together with increasing concentrations of PmxB. After injection and washing of column 1 for 10cvs, the bound material was eluted onto the reversed-phase column which was then eluted as in earlier experiments (Fig. 3). In the absence of PmxB, one peak eluted from the R2 and corresponded to the 9-mer(Rt=1.2 min; Fig. 3a, trace I). As the concentration of PmxB was increased, a decrease in the 9-mer peak was observed with concomitant increase in the PmxB peak (Fig. 3a; trade II). For clarity, Fig. 3a shows only those chromatograms obtained from incubating the lipopolysaccharide and 9-mer in the absence (trace I) and presence (trace II) of PmxB at the highest concentration (5 μM). Intermediate concentrations of PmxB produced a graded decline in the 9-mer peak. Amounts of the 9-mer and PmxB bound to lipopolysaccharide, when plotted against the concentration of PmxB (Fig. 3B), indicated that PmxB competes with binding of the 9-mer in a concentration-dependent fashion suggesting binding to the same region of lipopolysaccharide. Comparable results were obtained

when pentamidine competed with the 9-mer (data not shown). As expected, slightly lower concentrations of pentamidine were required to obtain the same level of displacement of the 9-mer from lipopolysaccharide. With the use of equation {4} from the Methods section, it is possible to use the information provided by the wash curves in Fig. 3 to determine k_{-1} values for each of the compounds tested (Table 1).

While the above data give an indication of the relative affinities of compounds for binding to lipopolysaccharide, they do not provide absolute values for the kds of these compounds. Saturation curves for increasing concentrations of PmxB and pentamidine binding to a fixed concentration of lipopolysaccharide revealed saturable binding (Fig. 4). Hill plots of the data obtained from these curves (Fig. 4 Inset) revealed a Hill coefficient (n_H) for pentamidine of 1.2 (correlation 0.99) and PmxB $n_H \sim 1.42$ (correlation 0.985) suggesting that there may be more than one binding site for the ligand on each molecule of lipopolysaccharide and only one ligand molecule may bind at a time, possibly due to steric hindrance.

It will be apparent to those skilled in the art that various modifications and variations can be made in the compounds and methods of the present invention without departing from the spirit or scope of the invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims and their equivalents.

What is claimed is:

1. A method for removing lipopolysaccharides, if present, from a sample comprising the steps of: (a) immobilizing an amidine containing moiety to a solid support such that it retains its ability to bind to lipopolysaccharides; (b) introducing a sample to the solid support under conditions sufficient to allow the lipopolysaccharides, if present, to bind to the amidine moiety.

2. The method of claim 1 wherein the solid support is selected from the group consisting of controlled pore glass, membranes, magnetic beads, magnetic particles porous polystyrene based supports, silica, silica gel, glass fibre frits and paper filters.

3. The method of claim 1 wherein the amidine moiety is True Blue.

4. The method of claim 1 wherein the amidine containing moiety is 4; 6-diamidino-2-Phenylindole.

5. The method of claim 1 wherein the support is suitable for perfusion chromatography.

6. The method of claim 1 wherein the sample comprises recombinant proteins.

7. The method of claim 1 wherein the sample comprises fluids for pharmaceutical preparation.

8. A method for detecting the absence, presence or concentration of a lipopolysaccharide in a sample comprising the step of: (a) contacting a sample with a detectable, modified amidine containing moiety, such that the molecule, if present, will form a complex with the amidine containing moiety thereby indicating the absence, presence or concentration of lipopolysaccharide.

9. The method of claim 8 wherein the amidine containing moiety is True Blue.

10. The method of claim 8 wherein the amidine moiety is DAPI.

11. The method of claim 8 wherein the sample comprises recombinant proteins.

12. The method of claim 8 wherein the sample comprises fluids for pharmaceutical preparations.

13. The method of claim 8 wherein the sample is obtained from a host organism.

14. A method for the purification of a sample preparation comprising immobilizing an amidine containing moiety to a solid support such that it retains its ability to bind to lipopolysaccharides; introducing the sample preparation to the support under conditions sufficient for lipopolysaccharides, if present, to bind to the immobilized amidine containing moiety; and, eluting the unbound components of the sample.

15. The method of claim 14 comprising the purification of recombinantly produced proteins.

16. An apparatus for the removal or detection of lipopolysaccharides from a sample comprising an amidine containing moiety immobilized to a solid support.

17. The apparatus of claim 16 wherein the solid support is selected from the group consisting of controlled pore glass, chips, membranes, magnetic beads, magnetic particles, porous polystyrene based supports, silica, silica gel, glass fibre frits and paper filters.

18. The apparatus of claim 17 wherein the support is suitable for perfusion chromatography.

19. The apparatus of claim 16 wherein the amidine containing moiety is True Blue.

20. The apparatus of claim 16 wherein the amidine containing moiety is 4'6-diamiclino-2-phenylindole.

21. A kit for the detection or removal of a lipopolysaccharide in a sample comprising: a solid support having immobilized thereon an amidine containing moiety capable of binding to lipopolysaccharide.

22. The kit of claim 21 wherein the solid support is selected from the group consisting of controlled pore glass, membranes, magnetic beads, magnetic particles, porous polystyrene based support, silica, silica gel, glass fibre frits and paper filters.

23. The kit of claim 22 wherein the support is suitable for perfusion chromatography.

24. The kit of claim 22 wherein the solid support is a column packed with particulate chromatography elements, said column being housed in a cartridge.

25. The kit of claim 24 wherein the cartridge is disposable.

26. The kit of claim 21 wherein the amidine containing moiety comprises a detectable moiety.

27. The kit of claim 26 wherein the modified amidine containing moiety is True Blue or DAPI.

28. A method for the removal a lipopolysaccharides from a solution comprising adding to the solution an amidine containing moiety capable of binding to the lipopolysaccharides with high affinity, wherein the complex formed by said binding has at least one physical property by which the complex can be separated from unbound components of the solution.

29. The method of claim 28 wherein bound complex, if formed, is separated from the unbound components of the solution by filtration, chromatography, or electrophoresis.

30. The method of claim 28 wherein the amidine containing moiety is DAPI or True Blue.

31. A method for the detection of a lipopolysaccharide in a sample comprising introducing to the sample an amidine containing moiety capable of binding to lipopolysaccharide thereby forming a detectable complex; and detecting said complex.

32. The method of claim 31 wherein the amidine containing moiety is True Blue or DAPI.

33. The method of claim 32 wherein the sample is selected from the group consisting of water, blood, cerebrospinal fluid, sweat, ascites fluid saliva, semen, amniotic fluid, and vaginal fluid.

34. A method of removing or inactivating endotoxins from a bodily fluid of a host organism comprising the steps of: (a) removing a fluid from the host; (b) contacting the fluid with an amidine containing moiety capable of binding to endotoxins with high affinity to form a complex; and, (3) separating the bound complex from the bodily fluid prior to reintroduction of the fluid into the host organism.

35. The method of claim 34 wherein the amidine containing moiety is DAPI or True Blue.

36. A method for detoxifying endotoxins in a host organism comprising the introduction into the organism of a therapeutically effective dose of a preparation comprising an amidine containing moiety capable of binding to endotoxins in vivo with high affinity, thereby inactivating the lipid A portion of the endotoxin.

37. The method of claim 36 wherein the amidine containing moiety is DAPI or True Blue.

38. A method of treatment for animals having an immunogenic reaction to a bacterial infection comprising the step of administering to the animal a therapeutically effective dose of a composition comprising an amidine containing moiety capable of inactivating the lipid A region of the endotoxins.

39. A pharmaceutical preparation for the treatment of organisms with endotoxin contaminants comprising a therapeutically effective amount of amidine containing moieties capable of binding to the endotoxins thereby rendering the endotoxins inactive.

40. A pharmaceutical preparation substantially pure of endotoxin contamination wherein said preparation was purified by contact with an amidine containing moiety immobilized to a solid support.

41. The method of claim 40 wherein the modified amidine containing moiety is DAPI or True Blue.

42. A method for the prevention of septic shock in animals caused by the presence of lipopolysaccharides, said method comprising administering to the animal a therapeutically effective dose of a composition comprising an amidine containing moiety capable of inactivating the lipopolysaccharide by binding to the lipopolysaccharide with high affinity.

43. A method for removing a phosphate-containing molecule from a sample comprising immobilizing an amidine containing moiety to a solid support; and introducing the sample to the support under conditions suitable for phosphate-containing molecules, if present, to bind to said amidine containing moiety thereby forming a complex.

44. The method of claim 47 wherein the phosphate-containing molecule is a nucleic acid or phosphoprotein.

45. The method of claim 1 wherein the amidine moiety is immobilized by a non-amidine containing linkage.

46. The method of claim 1 wherein the amidine containing moiety is a modified pentamidine.

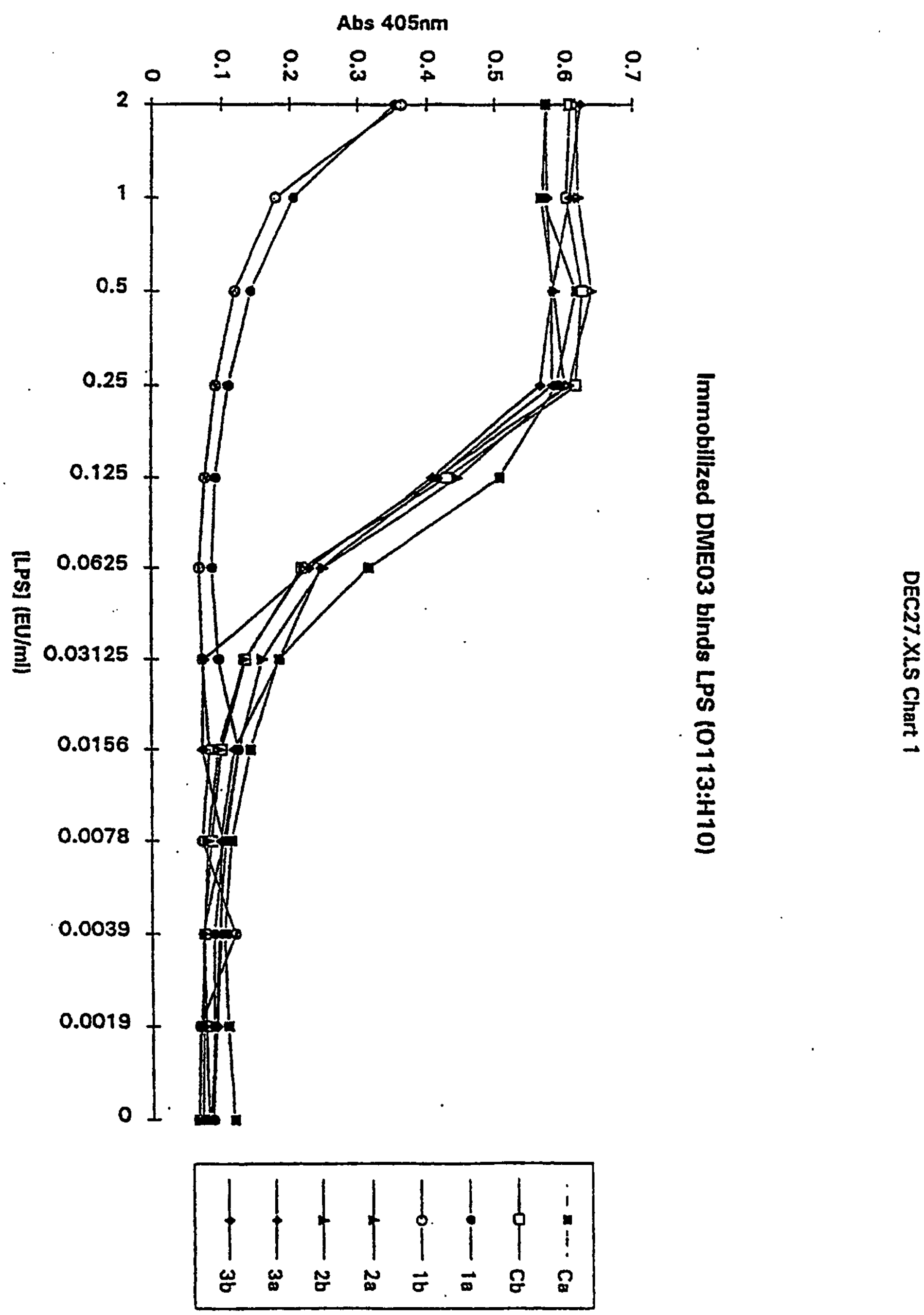
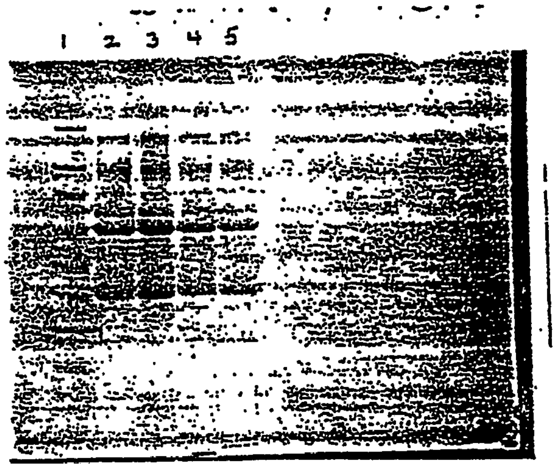
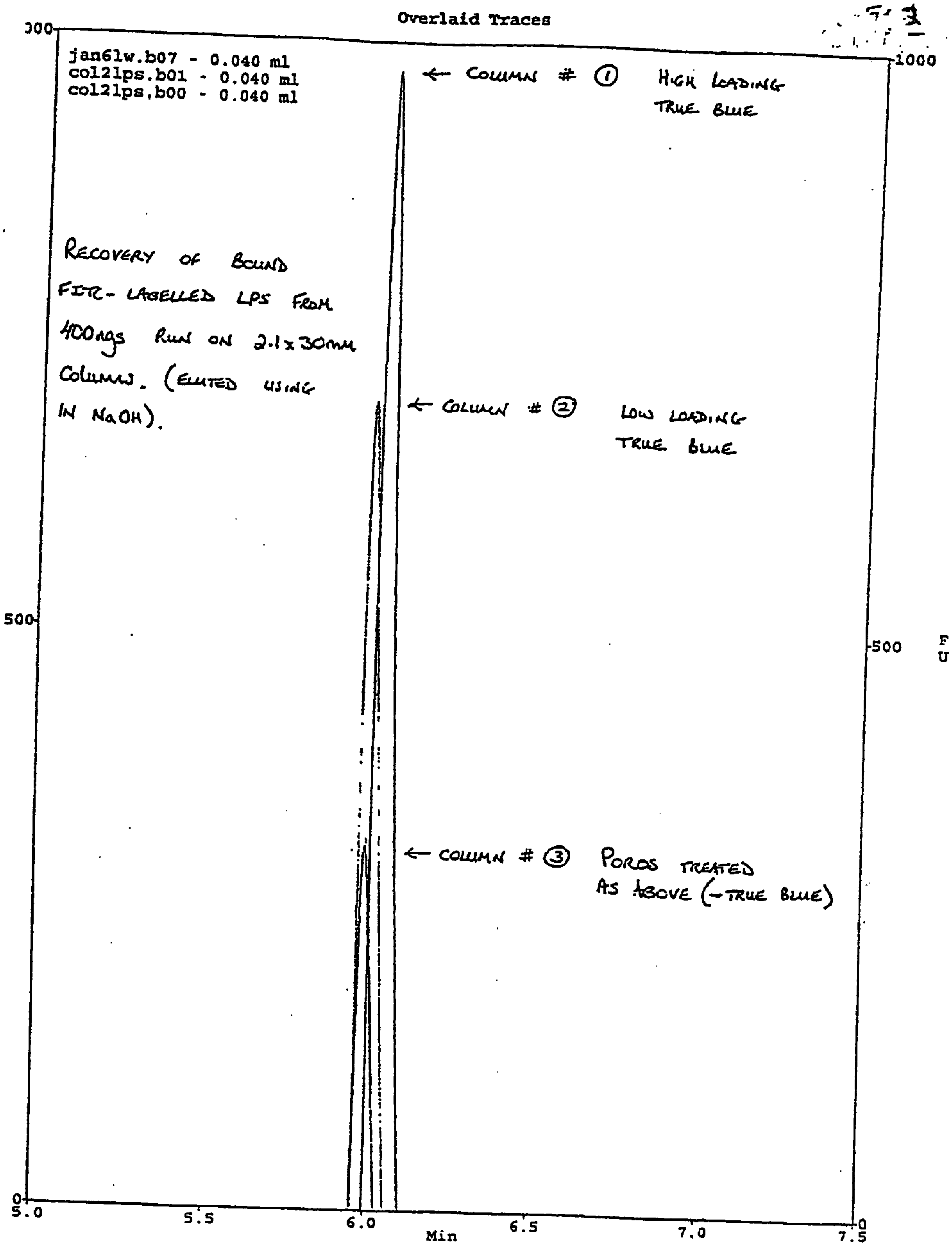
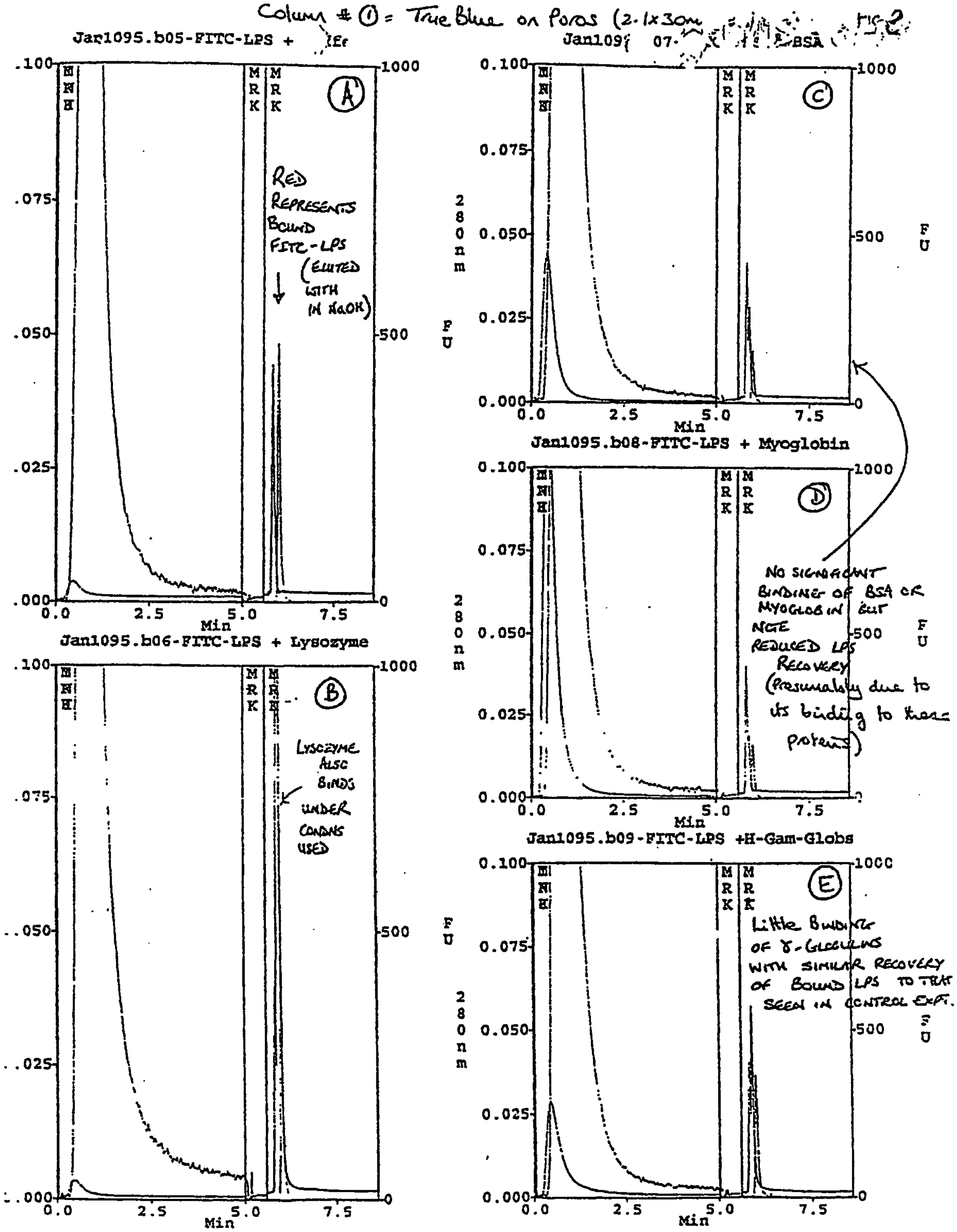

DEC27.XLS Chart 1
Immobilized DME03 binds LPS (O113:H10)
Abs 405nm
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
2
1
0.5
0.25
0.125
0.0625
0.03125
0.0156
0.0078
0.0039
0.0019
0
[LPS] (EU/ml)
Ca
Cb
1a
1b
2a
2b
3a
3b

Fig 1b





Overlaid Traces
jan61w.b07 - 0.040 ml
col21ps.b01 - 0.040 ml
col21ps.b00 - 0.040 ml
← COLUMN # 1 HIGH LOADING TRUE BLUE
RECOVERY OF BOUND FITC-LABELLED LPS FROM 400ngs RUN ON 2.1x30mm COLUMNS. (ELUTED USING 1N NaOH).
← COLUMN # 2 LOW LOADING TRUE BLUE
← COLUMN # 3 POROS TREATED AS ABOVE (-TRUE BLUE)
1000
500
0
500
FU
5.0
5.5
6.0
6.5
7.0
7.5
Min



RECOVERY OF FITC-LPS WHEN CO-INCUBATED WITH VARIOUS PROTEINS PRIOR TO CHROMATOGRAPHY OVER COLUMN # 1

JAN 1095 gsc

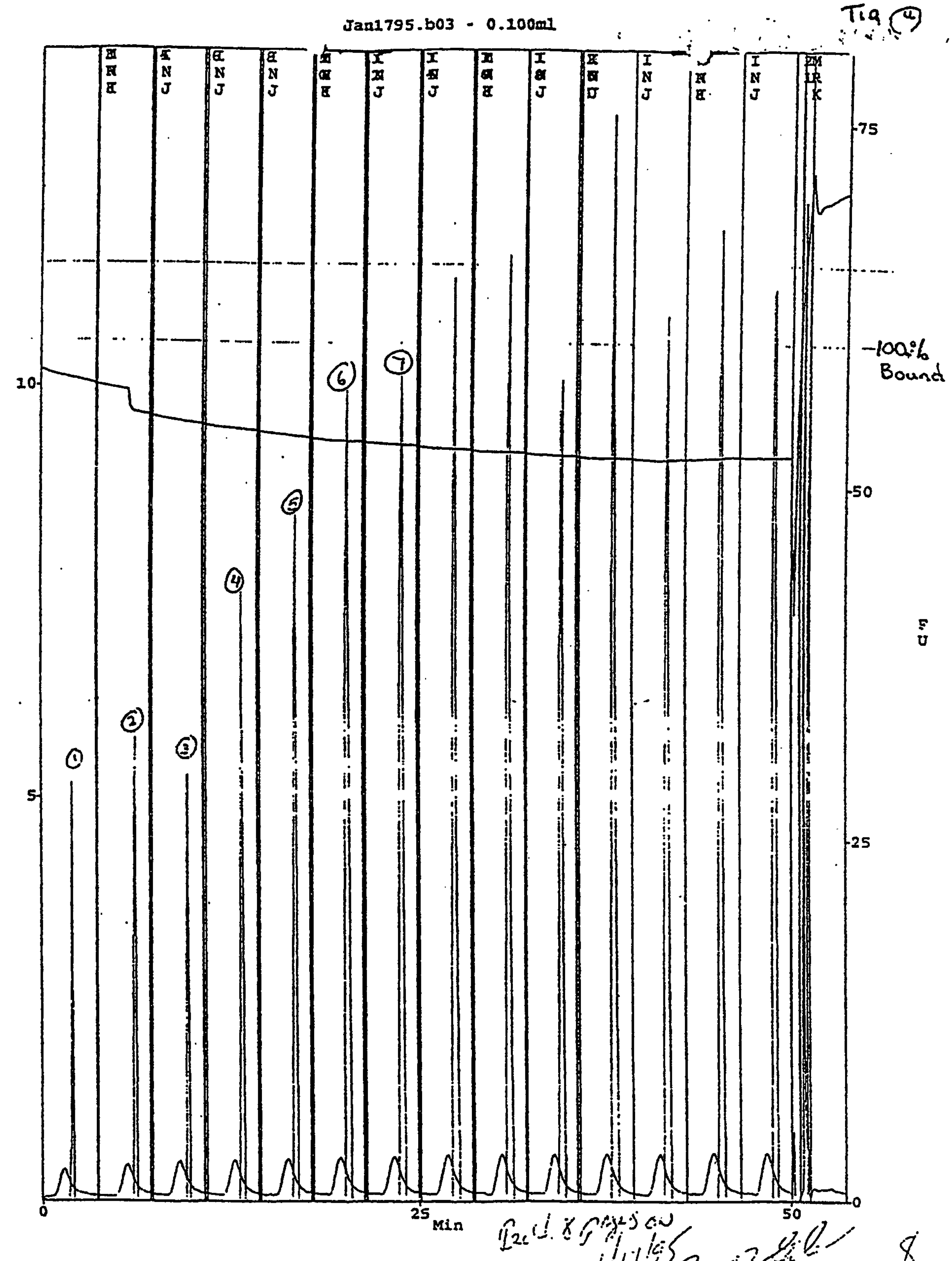


Fig (4)
Jan1795.b03 - 0.100ml
-100% Bound
10
5
0
25
Min
50
75
50
25
0
F
U

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/07475

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/569 G01N33/53 C07K1/22 A61K31/155

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,4 276 050 (FIRCA JOSEPH R ET AL) 30 June 1981 see the whole document ---	1,3-5,8, 13, 19-21, 23, 26-28, 30-33, 35,43,46
X	EP,A,0 376 480 (BECTON DICKINSON CO) 4 July 1990 see the whole document --- -/--	1,3,4,8, 13, 19-21, 26-28, 30-33, 35,43, 45,46

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
26 September 1996	07-10-1996
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Wells, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/07475

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,4 491 660 (GENDRICH RONALD L ET AL) 1 January 1985 see the whole document ---	1,2,5,8, 13,17, 21-23, 26,28, 29,43
A	EP,A,0 431 593 (DAICEL CHEM ;TANABE SEIYAKU CO (JP)) 12 June 1991 see page 8, line 9 ---	
X	GB,A,1 550 819 (NAT RES DEV) 22 August 1979 see the whole document ---	43,44
X	EP,A,0 221 308 (MILES LAB) 13 May 1987 see the whole document ---	43,44
X	EP,A,0 097 463 (BEECHAM GROUP PLC) 4 January 1984 see the whole document -----	39

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/07475

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:
   Remark : Although claims 36-38 and 42 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/07475

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		DE-T-	69010124	13-10-94
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		ES-T-	2068975	01-05-95
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Form PCT/ISA/210 (patent family annex) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/07475

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		US-A-	4539319	03-09-85
		US-A-	4594195	10-06-86

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